REMARKS

Favorable reconsideration, reexamination, and allowance of the present patent application are respectfully requested in view of the foregoing amendments and the following remarks.

The Rejection of Claim 1 Under 35 U.S.C. §112, 1st paragraph (new matter)

Claim 1 was rejected under 35 U.S.C. §112, first paragraph for allegedly containing subject matter which was not described in the specification in such a way to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner alleges that the specification does not provide support for proteins with an amino acid sequence having 80% or more homology with SEQ ID NO:2 that has no disclosed function. Applicants respectfully disagree with the Examiner's assertion; however, have amended the claims to add that the amino acid sequence of SEQ ID NO:2 also has an activity of binding to sucrose (see page 10, lines 7-11). The claims, as currently amended, clearly provide sufficient structural definition of the protein, as well as functional definition, such that the specification adequately describes that which applicants are claiming. Applicants assert that there is now sufficient structural and functional definition in the claims, and combined with the functional limitation regarding sucrose binding activity, applicant's assert that one skilled in the art is able to reasonable conclude that applicants were in possession of the claimed invention at the time the instant application was filed.

For these reasons, applicants respectfully request withdrawal of the rejection.

The Rejection of Claims 1 and 4-7 Under 35 U.S.C. §112, 1st paragraph (written description)

Claim 1 was rejected under 35 U.S.C. §112, 1st paragraph for allegedly containing subject matter which was not described in the specification in such a way to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner alleges that claim 1 encompasses a genus of proteins, including many functionally unrelated proteins.

Applicants respectfully disagree with the Examiner's allegations, however, in the interest of advancing prosecution, applicants have amended claim 1 to recite the sucrose binding activity of the claimed protein. Applicants point out to the Examiner that she indicated on page 2 of the Official Action that "the specification provides support for proteins with an amino acid sequence of 80% or more homologous to SEQ ID NO:2 and having sucrose binding activity". Therefore, applicants assert that the claimed invention is adequately described and supported.

Furthermore, the "Synopsis of Application of Written Description Guidelines", posted on the USPTO web site, state that a claim to a genus may be adequately described with only one exemplified species if a functional definition for a specific activity for the claimed species is present in the claim (see Example 14 "Product by Function", pages 53-55). This is the identical situation as to Example 14 in that a genus of a protein is claimed, and a specific function for that protein is also claimed. Therefore, the subject matter of the claim is adequately described.

For these reasons, applicants respectfully request withdrawal of the rejection.

Claims 4-7 were rejected under 35 U.S.C. §112, 1st paragraph for containing subject matter which was not described in the specification in such a way to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner alleges that while the hybridization conditions provide a structural limitation, the encoded proteins of claims 4-7 may have various activities other than binding to sucrose. The Examiner also alleges that the specification only discloses a single species of the claimed genus, nucleotides 3779-5761 of SEQ ID NO:1 encoding the sucrose binding enzyme II of the phosphotransferase system (PTSII) from *Brevibacterium lactofermentum* of SEQ ID NO:2.

Applicants respectfully disagree with the Examiner's allegations, however, claim 4 has been amended to add the functional limitation that the claimed DNA encodes a protein having an activity of sucrose binding enzyme II of the phosphotransferase system from *Brevibacterium lactofermentum.* Furthermore, the "Synopsis of Application of Written Description Guidelines", posted on the USPTO web site, state that a claim to a genus of DNAs may be adequately described with the recitation of stringent hybridization conditions if a specific activity for the encoded protein is present in the claim (see Example 9 "Hybridization", pages 35-37). Claims 4-7 represent the identical situation as to Example 9 in that a genus of a DNAs is claimed, and a stringent hybridization conditions are specified, as well as the specific function for the encoded protein. Therefore, the subject matter of the claim is adequately described.

For these reasons, applicants respectfully request withdrawal of the rejection.

The Rejection of Claims 1 and 4-7 Under 35 U.S.C. §112, 1st paragraph (enablement)

Claims 1 and 4-7 were rejected under 35 U.S.C. §112, 1st paragraph for allegedly failing to provide an enabling specification commensurate in scope with the claims. Applicants would like to point out that the rejection on page 5 of the Official Action is in direct contradiction with the statement on page 2, whereby the Examiner states "the specification provides support for proteins with an amino acid sequence of 80% or more homologous to SEQ ID NO:2 and having sucrose binding activity". The Examiner states on page 5 that the disclosure is only enabling for claims limited to a protein of SEQ ID NO:2 and a DNA of SEQ ID NO:1. This is a direct contradiction, and applicants request clarification. Applicants will completely address this rejection, however, in the interest of advancing prosecution.

As stated above, applicants have amended the claims to add the function of the encoded protein, and specifically in claim 4, the specific activity of the protein from *Brevibacterium lactofermentum*. It is asserted that the claims are now commensurate with the enablement provided by the disclosure in that the number of protein and DNA species encompassed by the claims are of a number that is reasonable when evaluated under the Wands factors, particularly in view of the predictability in the art, and the abundant guidance in the specification (see page 9, line 21 – page 11, line 10, and the examples), and the functional definitions now present in the claims. Applicants assert that the experimentation necessary to practice the invention is not undue in light of the breadth of the claims as amended and in view of the disclosure.

Regarding the Examiner's assertion that the specification fails to provide

information regarding mutations which could be made in the DNA sequence, and retain the ability to encode a protein with the requisite activity, applicants have provided evidence in the form of the attached literature reference, Chen et al. *Infection and Immunity*, 61:2602-2610 (1993). This reference shows functional domains of sucrose binding specific enzyme II (EII^{suc}). Specifically, figure 6 indicates amino acid sequences of EII^{suc} from *Streptococcus sobrinus* and *Streptococcus mutans* and consensus sequences of these proteins. These alignments indicate 39% identity in the N-terminus region of 460 amino acids (see 2607, left column, 1st paragraph). Such comparisons indicate that an upstream consensus sequence exists and therefore, indicates a functional domain.

Such information provides guidance to the skilled art worker in determining which domains of the claimed DNA/protein are amenable to change or mutation, while still retaining sucrose binding activity.

In light of the amendments to the claims and the above arguments, applicants assert that these rejections are moot, and respectfully request they be withdrawn.

Att'y Dkt. No.
US-1380

U.S. App. No: 10/019,284

Conclusion

For at least the foregoing reasons, Applicant respectfully submits that the present patent application is in condition for allowance. An early indication of the allowability of the present patent application is therefore respectfully solicited.

If Examiner Slobodyansky believes that a telephone conference with the undersigned would expedite passage of the present patent application to issue, she is invited to call on the number below.

It is not believed that extensions of time are required, beyond those that may otherwise be provided for in accompanying documents. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and the undersigned hereby authorizes any such charges to be charged to the credit card recited in the attached PTO-2038.

Respectfully submitted,

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INFECTION AND IMMUNITY, June 1993, p. 2602–2610
0019-9567/93/062602-09$02.00/0

Vol. 61, No. 6

Sequence Analysis of *scrA* and *scrB* from *Streptococcus sobrinus* 6715

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Received 19 November 1992/Accepted 16 March 1993

The complete nucleotide sequences of *Streptococcus sobrinus* 6715 *scrA* and *scrB*, which encode sucrose-specific enzyme II of the phosphoenolpyruvate-dependent phosphotransferase system and sucrose-6-phosphate hydrolase, respectively, have been determined. These two genes were transcribed divergently, and the initiation codons of the two open reading frames were 192 bp apart. The transcriptional initiation sites were determined by primer extension analysis, and the putative promoter regions of these two genes overlapped partially. The gene encoding enzyme II^{Scr}, *scrA*, contained 1,896 nucleotides, and the molecular mass of the predicted protein was 66,529 Da. The hydropathy plot of the predicted amino acid sequence indicated that enzyme II^{Scr} was a relatively hydrophobic protein. The gene encoding sucrose-6-phosphate hydrolase, *scrB*, contained 1,437 nucleotides. The molecular mass of the predicted protein was 54,501 Da, and the encoded enzyme was hydrophilic. The predicted amino acid sequences of the two open reading frames exhibited approximately 46 and 70% identity with those encoded by *scrA* and *scrB*, respectively, from *Streptococcus mutans* GS5. Homology also was observed between the N-terminal region of the *S. sobrinus* 6715 enzyme II^{Scr} and other enzyme IIs specific for the glucopyranoside molecule, all of which generate glucopyranoside-6-phosphate during translocation and phosphorylation of the respective substrates. The sequence of the C-terminal domain of the *S. sobrinus* 6715 enzyme II^{Scr} shared significant homology with enzyme III^{Glc} from *Escherichia coli* and *Salmonella typhimurium* and with the C-terminal domain of enzyme II^{Bgl} from *E. coli*, indicating that the two functional domains, enzyme II^{Scr} and enzyme III^{Scr}, were covalently linked as a single polypeptide in *S. sobrinus* 6715. The deduced amino acid sequence of the gene product of *S. sobrinus scrB* shared strong homology with sucrase from *Bacillus subtilis*, *Klebsiella pneumoniae*, and *Vibrio alginolyticus*, suggesting conservation based on the physiological roles of these proteins.

Mutans streptococci (MS) have been identified as the principal etiological agents of dental caries, and the significance of sucrose metabolism by these organisms in their ability to initiate tooth decay has been demonstrated (13, 15, 25). Sucrose is the substrate for glucosyltransferase and fructosyltransferase, which synthesize glucan and fructan polymers, respectively. The formation of extracellular glucans enhances the colonization of these organisms on the tooth surface (15, 25), whereas the synthesis of fructans provides an extracellular carbon source which can be metabolized during periods of starvation (3, 42). However, only a small percentage of available sucrose is processed to produce exopolymers; most of the sucrose in the environment is efficiently transported into and metabolized by MS.

The primary mechanism for sucrose uptake by MS, at least at low substrate concentration, is the high-affinity phosphoenolpyruvate-dependent phosphotransferase system (sucrose-PTS; 19, 27, 43–45), by which sucrose is concomitantly transported and phosphorylated by the activity of a membrane-bound permease, the sucrose-specific enzyme II (EII^{Scr}). The phosphorylated sucrose is then hydrolyzed by sucrose-6-phosphate hydrolase, yielding glucose-6-phosphate and fructose (4, 46). The metabolism of these two monosaccharides via glycolysis results in the production of lactic acid. In the past decade, much attention has been focused on the analysis of the mechanisms of sucrose metabolism in MS. More recently, the genes encoding EII^{Scr}, *scrA*, and sucrose-6-phosphate hydrolase, *scrB*, have been cloned from two human cariogenic pathogens, *Streptococcus mutans* GS5 (17, 26, 39) and *Streptococcus sobrinus* 6715 (5). The nucleotide sequences of both genes from *S. mutans* GS5 have been reported (38, 39). The *scrA* and *scrB* of *S. mutans* GS5 are adjacent to each other on the chromosome (39) and are transcribed divergently. The deduced amino acid sequence of the N terminus of *S. mutans* GS5 EII^{Scr} shares homology with EIII-dependent EII^{Scr} found in *Bacillus subtilis* and *Escherichia coli*, and the C-terminal sequences share homology with $EIII^{Glc}$ of *Salmonella typhimurium* and the C terminus of *E. coli* EII^{Bgl} (39). These results suggest that the sucrose PTS-specific component of *S. mutans* GS5 is EIII independent (39). Homology also was detected between *S. mutans* GS5 *scrB* and *B. subtilis sacA*, both genes encoding enzymes that use sucrose as the substrate (38). Comparable genetic information has not been reported for *S. sobrinus* 6715.

In a previous study, we suggested that a 4.2-kb DNA fragment isolated from *S. sobrinus* 6715 genomic DNA partially digested with *Sau*3A may contain both *scrA* and *scrB*. This observation was based on the expression of EII^{Scr} and sucrose-6-phosphate hydrolase activities by *Lactococcus lactis* subsp. *lactis* LM0230 transformants harboring a plasmid onto which this fragment had been subcloned and on the ability of such transformants to grow at the expense of sucrose (5). In this study, we present the results of nucleotide sequence analyses of the chromosomal region of *S. sobrinus* 6715 containing *scrA* and *scrB*.

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MATERIALS AND METHODS

Bacterial strains, media, and reagents. Two *L. lactis* subsp. *lactis* LM0230 transformants (MC127-1 and MC 127-2), which harbor a 4.2-kb *S. sobrinus*-derived DNA fragment encoding EII^{Scr} and sucrose-6-phosphate hydrolase, were described previously (5). The 4.2-kb DNA fragment was cloned in opposite orientations in relation to the vector sequence in these two transformants. These strains were grown routinely at 37°C in a chemically defined medium (FMC [47]) with 0.5% NZ amine (ICN Nutritional Biochemicals, Cleveland, Ohio) substituting for all amino acids, 10 mM sucrose, and spectinomycin at 500 μg/ml. *E. coli* strains were grown in LB medium (36) containing, when indicated, spectinomycin (75 μg/ml) or ampicillin (50 μg/ml). All chemical reagents and antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.) and ICN Biochemicals Inc. (Irvine, Calif.), respectively. All restriction endonucleases were obtained from Life Technologies, Inc. (Gaithersburg, Md.). Alkaline phosphatase from calf intestine, nuclease S1, polynucleotide kinase, Klenow fragment of DNA polymerase I, T4 DNA ligase, and Moloney murine leukemia virus reverse transcriptase also were purchased from Life Technologies, Inc. α-^{35}S-dATP (3,000 Ci/mmol) and [γ-^{32}P]ATP (4,500 Ci/mmol) were purchased from NEN Research Products (Boston, Mass.) and ICN Biochemicals Inc., respectively.

Plasmid construction, DNA purification, and DNA sequencing. DNA fragments internal to the 4.2-kb fragment obtained after partial digestion with *Sau*3A and the 2.7-kb fragment obtained after complete digestion with *Hin*dIII of *S. sobrinus* 6715 genomic DNA (5) were subcloned onto pGEM2f vectors (Promega Corp., Madison, Wis.) or pDL278 (21, 22) to generate single- or double-stranded DNA for sequencing. Nested deletions of appropriate double-stranded fragments were obtained by the method of Henikoff (18). Nucleotide sequences were determined by the dideoxy chain termination method (37) with DNA templates from single- and double-stranded phagemid DNA or double-stranded plasmid DNA purified from *E. coli* as described before (6, 50). Sequencing reactions were initiated from the pUC/M13 17-mer universal forward sequencing primer or the reverse sequencing primer, using α-^{35}S-dATP. The sequences of both DNA strands were obtained. DNA sequence analyses were performed with the MicroGenie program (Beckman Instruments, Palo Alto, Calif.) and Genetics Computer Group (University of Wisconsin, Madison) package (7).

RNA isolation. Total cellular RNA from *L. lactis* subsp. *lactis* LM0230 transformants was isolated as described by Galli et al. (12) with modifications. Bacteria were grown at 37°C to early-exponential phase in FMC medium (47) with 10 mM L-threonine, 10 mM sucrose, and spectinomycin at 500 μg/ml. Cells were treated with lysozyme (5 mg per 50-ml original culture volume) for 5 min at 37°C, and 10% sodium dodecyl sulfate was added to a final concentration of 0.8%. Total cellular RNA was then purified from the lysate (12).

Primer extension analysis. The *scrA* and *scrB* transcriptional start sites were determined by primer extension analysis, using oligonucleotides described in Results. The procedures and solutions used were those of Yeung (50) with modifications. Fifty micrograms of total cellular RNA was used in each reaction. The mixture of RNA and the 5'-end-labeled γ-^{32}P-oligonucleotide was heated at 95°C for 30 s and then incubated at 37°C (primers A1, B1, and B2) or 42°C (primer A2) for 30 min to allow complete annealing. The synthesis of a cDNA strand was carried out in 50 mM Tris-HCl (pH 8.3)–75 mM KCl–3 mM $MgCl_2$–10 mM dithiothreitol, with 200 U of Moloney murine leukemia virus reverse transcriptase and 10 mM deoxynucleoside triphosphate. Products extended were analyzed along with a DNA sequencing reaction, using the same primer on a 6% polyacrylamide gel.

Nucleotide sequence accession numbers. The sequences of *scrA* and *scrB* from *S. sobrinus* 6715 were submitted to GenBank and assigned accession numbers L06791 and L06792, respectively.

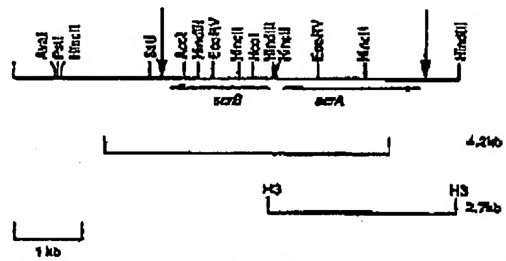


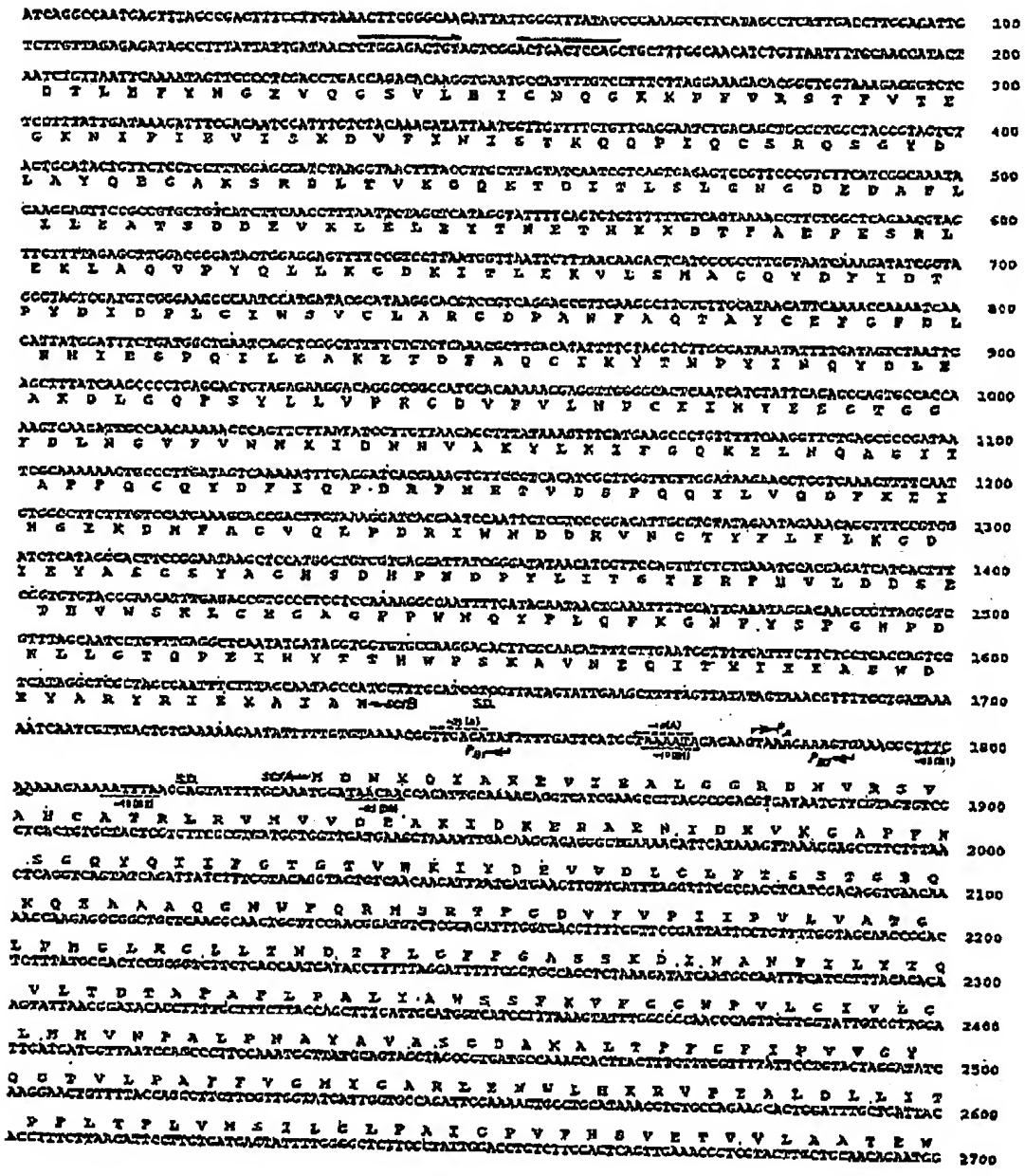
FIG. 1. *scr* region of the *S. sobrinus* 6715 chromosome. A restriction endonuclease map of the chromosomal region containing *scrA* and *scrB* is shown on the top line. The relative locations and transcriptional directions of *scrA* and *scrB* are indicated by horizontal arrows. The limits of the DNA sequence shown in Fig. 2 are indicated by vertical arrows. The 4.2- and 2.7-kb DNA fragments cloned directly from *S. sobrinus* 6715 genomic DNA, and from which subclones were obtained for sequencing purposes, are indicated below the map. H3, *Hin*dIII.

RESULTS

Localization of *scrA* and *scrB* on the *S. sobrinus* chromosome. The nucleotide sequence of the 4.2-kb DNA fragment revealed one complete open reading frame (ORF: ORF1) and the 5' end of a partial ORF (ORF2). The sequence of ORF2 was then completed with sequences from the isolated 2.7-kb *Hin*dIII fragment which overlapped the 4.2-kb DNA fragment (Fig. 1). The two ORFs were separated by 192 bp (nucleotides 1639 to 1830) and transcribed divergently (Fig. 2). Because sequence homology existed between ORF1 and *S. mutans* GS5 *scrB* and between ORF2 and *S. mutans* GS5 *scrA*, ORF1 and ORF2 were designated *scrB* and *scrA*, respectively.

Nucleotide sequence analysis of *scrB*. ORF1 (*scrB*) contained 1,437 bp, similar to *S. mutans* GS5 *scrB* (1,363 bp [38]). Approximately 70% sequence identity was observed between these two alleles. The complete DNA sequence of *scrB* and the deduced amino acid sequence are presented as the strand complementary to the reading strand in Fig. 2. ORF1 began with an ATG initiation codon (nucleotide 1638) and ended with a TAG termination codon (nucleotide 201) that encoded a protein with a calculated molecular weight of 54,501. The potential *E. coli* consensus Shine-Dalgarno sequence, AGGAGG (nucleotides 1653 to 1648), was located 9 bases 5' to the ATG codon.

The transcriptional initiation site for *scrB* was identified by primer extension analysis, using two oligonucleotides. Primer B1, 5'-GGCTCAATATGATAGGTG-3' (nucleotides 1594 to 1610), is located 29 bases and primer B2, 5'-CCAGTCCTCATAGGCTC-3' (nucleotides 1520 to 1537), is located 102 bases 3' to the translational start site. Two major



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 I  L  A  L  P  F  G  I  A  G  I  I  I  G  G  L  Q  Q  V  I  V  V  T  G  V  H  H  I  F  N  F  L  E
ATTCTTGCTTTGCCATTTGGTATTGCAGGTATCATTATCGGCGGTCTGCAACAAGTTATTGTTGTTACCGGAGTTCACCACATCTTTAATTTCCTTCAAA  2800
 T  Q  L  L  A  I  T  K  A  N  P  F  N  P  L  L  S  A  A  T  A  G  Q  V  G  A  N  L  A  V  A  V  K  T
CTCAGCTTTTAGCTCAAACAAAGCCCAATCCATTTAACCCACTTCTTTCTGCGGCTACTGCTGGTCAGGTCGGCGCCAACCTTGCCGTAGCTGTTAAAAC  2900
 K  S  A  K  L  K  A  L  A  Y  P  S  A  L  S  A  A  L  G  I  T  E  P  A  I  F  G  V  N  L  R  Y  G
AAAGAGTGCCAAACTTAAAGCTCTTGCTTATCCATCTGCACTTTCTGCGGCTCTTGGGATTACAGAACCGGCTATCTTCGGCGTTAACCTTCGTTATGGA  3000
 K  P  F  V  M  G  L  V  G  G  S  A  G  G  P  I  A  A  L  V  G  L  K  A  T  G  M  S  V  T  V  L  P
AAACCATTTGTTATGGGCCTTGTCGGTGGTTCAGCTGGTGGATTCATCCCACCTCTTGTAGGCCTTAAGGCGACAGGGATGTCTGTAACCGTTCTTCCTC  3100
 G  L  L  L  F  L  N  S  Q  M  P  M  Y  I  V  G  I  T  V  A  C  A  I  A  F  A  L  T  Y  Y  F  G  Y  A
GACTTCTCCCTCTTCCTTAATTCTCAAATGCCAATGTATATTGTTTCCATCACAGTAGCCTGTGCTATCGCTTTCGCTTTGACATATTACTTCGGCTATGC  3200
 D  K  E  E  D  V  S  A  K  K  P  E  A  P  A  A  A  P  V  A  E  T  E  T  K  S  E  V  I  A  E  P  L
GGATAAGGAGGAAGACGTATCTGCAAAAAAGCCTGAAGCGCCTGCGGCAGCACCGGTAGCTGAAACTGAAACAAAATCAGAAGTTATTGCAGAGCCTCTT  3300
 D  G  E  A  V  E  L  S  K  V  N  D  P  V  F  S  E  E  A  M  G  K  G  I  A  V  K  P  E  G  N  T  V
GACGGTGAAGCTGTAGAGCTTTCTAAGGTAAATGATCCTGTCTTTTCTGAGGAAGCTATGGGCAAAGGCATTGCTGTCAAACCTGAGGGCAATACTGTTT  3400
 Y  S  P  V  N  G  T  V  Q  I  A  F  E  T  G  H  A  Y  G  L  K  S  D  N  G  A  E  V  L  I  H  V  G  I
ATTCACCCGTTAATGGTACGGTTCAGATTGCTTTTGAAACAGGTCATGCTTACGGTCTTAAATCGGACAATGGCGCAGAAGTCCTGATCCATGTCGGCAT  3500
 D  T  V  G  M  N  G  T  G  F  D  Q  K  V  A  A  N  Q  T  V  K  V  G  D  V  L  G  T  F  D  S  A  K
TGATACCGTTTCTATGAATGGAACAGGATTTGATCAAAAGGTTGCTGCTAACCAGACTGTTAAAGTCGGTGATGTTCTTGGAACCTTTGACTCTGCAAAA  3600
 I  A  E  A  G  L  D  D  T  T  M  V  I  I  T  N  T  A  D  Y  S  E  V  K  P  L  A  A  G  Q  L  A  M
ATTGCAGAAGCTGGTCTTGATGATACAACAATGGTCATCATCACCAATACAGCAGATTATTCAGAAGTTAAACCACTTGCTGCTGGTCAGTTGGCGATG  3700
 G  A  D  L  L  E  L  N  K
GGGCAGAGCTTTTGGAACTGAACAAATAAAGCTTAACAAACCTCAGAAAGAACTCAGCTTTCTTGATTTAGCTATGGCTTTGTCATCTTAAACCCAGAAG  3800
TCAAAAAAGCCTGGATAGGATTGCTTTCTGCCCTTATTTTTCTTATAATGTTAGTAAGATTTTAACACAAGGAGACGTCTATGTCTAAACTGTATGGAAG  3900
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FIG. 2. Nucleotide and deduced amino acid sequences of the *scrA* and *scrB* region of *S. sobrinus* 6715. The relative location of this sequence on the chromosomal map is indicated in Fig. 1. The two ORFs were 192 bp apart and transcribed divergently from the opposite DNA strands. Therefore, the sequence of ORF1 (nucleotides 1638 to 202; *scrB*) presented here is the noncoding strand, and the sequence of ORF2 (nucleotides 1831 to 3726; *scrA*) is the coding strand. Amino acids of the gene products are provided in standard one-letter code below their anticodons for ORF1 and above their codons for ORF2. The putative Shine-Dalgarno site for each gene is indicated as S.D. with the appropriate bases in bold letters. The transcriptional start sites determined by primer extension analyses (Fig. 3) for each gene are indicated as P_A, P_{B1}, and P_{B2}, and the corresponding −10 and −35 regions are overlined (P_A) or underlined (P_{B1}) and P_{B2}). The inverted repeats of proposed rho-independent terminators are shown by inverted arrows.

signals, 114 bases (P_{B1}) and 152 bases (P_{B2}) 5′ to the ATG site, corresponding to the T residue and the G residue at nucleotides 1753 and 1790, respectively, were observed consistently with primer B1 with total cellular RNA isolated from MC127-1 and MC127-2 (Fig. 3B). No signal was detected when primer B2 was used. Two possible *E. coli* σ^{70}-type promoter regions corresponding to each signal were located 14 bases 5′ to P_{B1}, TTGAAA-N_{22}-TATTTT, and 19 bases 5′ to P_{B2}, TTGTTA-N_{20}-TAAATT (Fig. 2) (16). The sequences of these two putative promoter regions are homologous to the *E. coli* consensus, but the spacing between −10 and −35 sequences is rather long (16). An inverted repeat which may be the transcriptional terminator for *scrB* was observed 3′ to the *scrB* termination codon (nucleotides 136 to 146 and 153 to 163). The calculated free energy of this stem-loop structure was −6 kcal (ca. −25 kJ)/mol.

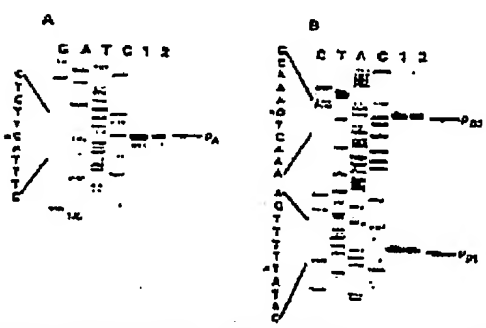

FIG. 3. Primer extension analysis of *scrA* (A) and *scrB* (B) transcripts. RNA was isolated from MC127-1 (lane 1) and MC127-2 (lane 2). Radiolabelled oligonucleotides were incubated with the RNA, and the cDNA was synthesized by the activity of reverse transcriptase as described in Materials and Methods. The same oligonucleotides were used to prime dideoxy sequencing products from a DNA template that contained the *scrA* and *scrB* junction region.

The hydropathy plot (20) of the deduced amino acid sequence of *scrB* (Fig. 4B) indicated that the protein was relatively hydrophilic. The amino acid composition of this protein suggested that it was slightly acidic (15.1% acidic versus 8.7% basic amino acids).

Comparison of *S. sobrinus* 6715 sucrose-6-phosphate hydrolase with other proteins. The deduced amino acid sequence of sucrose-6-phosphate hydrolase from *S. sobrinus* 6715 was compared with that from *S. mutans* GS5; 70% sequence homology was observed between these two proteins (Fig. 5). This suggested that these two polypeptides may have been derived from a common ancestor. Extensive homology was observed throughout the entire protein except for a stretch of 25 amino acids (amino acids 187 to 211) that was found in the *S. sobrinus* 6715-encoded protein but not in the protein encoded by *S. mutans* GS5. The primary structure of *S. sobrinus* 6715 sucrose 6-phosphate hydrolase was compared with those of other enzymes exhibiting sucrose hydrolytic

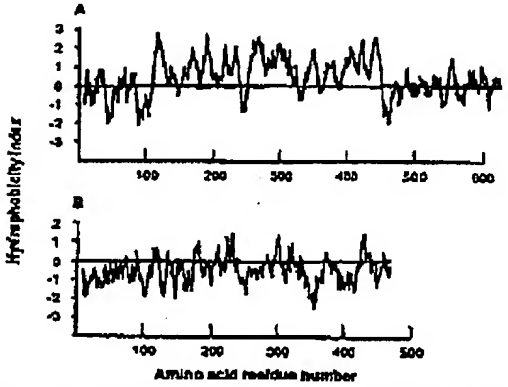

FIG. 4. Kyte and Doolittle (20) hydropathy plot of the EII^{Scr} (A) and sucrose-6-phosphate hydrolase (B) predicted by *scrA* and *scrB*, respectively, of *S. sobrinus* 6715.

activity. Sequence identity of approximately 35% to the entire sucrase sequences of *Vibrio alginolyticus* (41), *Klebsiella pneumoniae*, and *B. subtilis* was detected (10). Lesser homology was observed with *E. coli* sucrose hydrolase (28%) (1) and *Zymomonas mobilis* sucrase (25%) (14).

Nucleotide sequence analysis of *scrA*. The partial sequence of the 2.7-kb *Hin*dIII fragment, which hybridizes to *S. mutans* GS5 *scrA* under conditions of low stringency and was described previously (5), revealed an ORF, *scrA*, of 1,896 bp encoding a 632-amino-acid protein with a calculated molecular weight of 66,529. Approximately 60% homology was observed between the two alleles from *S. mutans* GS5 and *S. sobrinus* 6715. The complete DNA sequence of *scrA* (ORF2) and the predicted amino acid sequence of the putative protein product are shown in Fig. 2. ORF2 started at an ATG (nucleotide 1831) and ended at a TAA (nucleotide 3727). A potential *E. coli* Shine-Dalgarno sequence, AGGAG, was found 10 nucleotides 5′ (nucleotides 1817 to 1820) to the ATG start codon.

The 5′ end of the mRNA transcribed from *scrA* was mapped by primer extension analysis, using two oligonucleotides complementary to the coding strand. These two primers, A1 (5′-CGCCTAAGGCTTCGATGA-3′) and A2 (5′-CCTGAGTTAAAGAAGGCTCC-3′), were located 29 and 156 bases 3′ to the translational start site, respectively. The 5′ end of the transcript was identified by using both primers with total cellular RNA isolated from MC127-1 and MC127-2 (5). The cDNA extension product indicated that the G residue (P_A, nucleotide 1779) located 51 bases 5′ to the ATG start site was the transcriptional initiation site for *scrA* (Fig. 3A). The promoter-like sequence TTGACA-N_{16}-TAAAAT indicated in Fig. 2 was located 6 bp 5′ to the G (P_A) (16). Two inverted repeats which may function as the transcriptional termination sites of *scrA* were observed 5 and 45 bases 3′ to the TAA termination codon. The estimated free energy values of these secondary structures were −20 kcal (ca. −84 kJ)/mol and −9.8 kcal (ca. −41 kJ)/mol, respectively.

The predicted EII^{Scr} contained 52% hydrophobic, 31% hydrophilic, 8% positively charged, and 9% negatively charged amino acids. Thus, EII^{Scr} appeared to be a relatively hydrophobic protein. These 106 charged amino acids were concentrated mostly in the N-terminal 110 amino acids (containing 32 charged amino acids) and C-terminal 122 amino acids (containing 42 charged amino acids), indicating that they are the hydrophilic regions. The hydropathy plot (20) of the deduced amino acid sequence of *scrA* (Fig. 4A), confirmed that the hydrophobic domain was located between amino acids 110 and 455. Several prominent hydrophilic domains were identified in this region, suggesting that it likely contains more than one transmembrane domain.

Comparison of *S. sobrinus* 6715 EII^{Scr} with other proteins. The deduced amino acid sequence of EII^{Scr} from *S. sobrinus* shared 43% identity with the *S. mutans* EII^{Scr} (39) (Fig. 6)

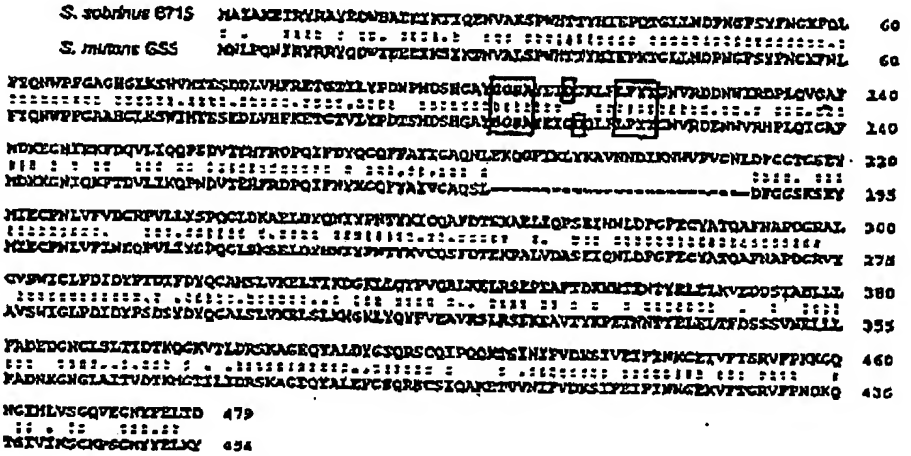

FIG. 5. Comparison of sucrose-6-phosphate hydrolase from *S. sobrinus* 6715 and *S. mutans* GS5. Amino acid sequences are presented in one-letter standard codes and have been aligned by introducing gaps (hyphens) to maximize identities. Identical residues are indicated by colons, and conserved substitutions are shown by dots. The numbers at the end of each line represent the positions of the amino acid in each sequence. The sucrose box is boxed.

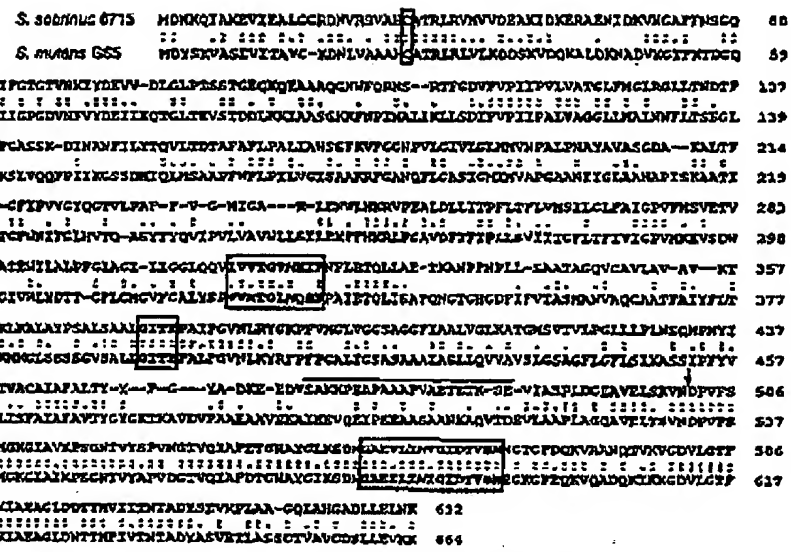

FIG. 6. Comparison of EII^{Scr} from *S. sobrinus* 6715 and *S. mutans* GS5. Amino acid sequences are presented and have been aligned as in the legend to Fig. 4. The conserved Cys-26, the GITE motif, and the conserved amino acid sequence are boxed. The histidyl residues within the consensus sequences are in italics. The proposed Q-linker region is overlined. The vertical arrow indicates the limit of the predicted amino acid sequence of the 4.2-kb *S. sobrinus* 6715-derived DNA fragment.

and 39, 36, and 36% identity with the N-terminal 460 amino acids of *E. coli* (8, 40), *V. alginolyticus* (2), and *B. subtilis* EII^{Scr} (11), respectively. On the other hand, the C-terminal 170 amino acids of *S. sobrinus* EII^{Scr} exhibited 37% homology with $EIII^{Glc}$ of *E. coli* and *Salmonella typhimurium* (28). These data indicated that EII^{Scr} of *S. sobrinus* 6715, like that of *S. mutans* GS5 (39), was an EIII-independent protein in which the EII and EIII functional domains are covalently linked as a single polypeptide.

DISCUSSION

The isolation in *E. coli* and subsequent transfer to *L. lactis* subsp. *lactis* LM0230 of genes encoding EII^{Scr} (*scrA*) and sucrose-6-phosphate hydrolase (*scrB*) from *S. sobrinus* 6715 was described previously (5). In this study, the nucleotide sequences of both genes were determined. The two genes were located adjacent to each other on the *S. sobrinus* 6715 chromosome and were transcribed divergently. A similar arrangement was reported in *S. mutans* GS5 (39). These data would suggest that the two genes are not regulated as a single operon in MS. However, the corresponding genes of *B. subtilis* and *E. coli* are adjacent to each other on the respective chromosomes and are transcribed in the same direction (8, 11). The arrangement of these two genes in *S. sobrinus* 6715 is compatible with previous observations that the activity of EII^{Scr} is inducible while the activity of sucrose-6-phosphate hydrolase is constitutive (45, 46).

A comparison of the amino acid sequences of sucrose-6-phosphate hydrolase (ScrB) from *S. sobrinus* 6715 and from *S. mutans* GS5 indicated that these two proteins were highly conserved. The sucrose box, a consensus sequence containing nine amino acids proposed by Sato and Kuramitsu (38), and highly conserved among four sucrose hydrolytic enzymes, also was identified in *S. sobrinus* 6715 sucrose-6-phosphate hydrolase. We extended the comparison to other sucrose hydrolytic enzymes and found that the sucrose box is highly conserved among all sucrose hydrolytic enzymes examined (Fig. 7). A stretch of 25 amino acids found in the predicted *scrB* product of *S. sobrinus* 6715 but not in that of *S. mutans* GS5 (Fig. 5) may have been the result of insertion or deletion during evolution.

Sequence comparisons among different EII and EIII proteins demonstrate that EII complexes, the permeases of the PTS, generally consist of three structurally distinct domains (EIIA, -B, and -C) which together form a functional unit (34). The major hydrophilic and hydrophobic functional domains contain highly conserved consensus sequences for phosphorylation and interdomain interaction, although their arrangements may differ among the proteins studied (23, 24, 33–35).

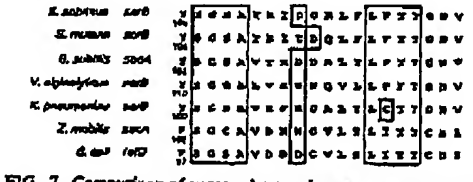

FIG. 7. Comparison of sucrose box regions among seven sucrose hydrolytic enzymes. *S. sobrinus scrB* and *S. mutans scrB* encode sucrose-6-phosphate hydrolase; *B. subtilis sacA*, *V. alginolyticus scrB*, *K. pneumoniae scrB*, and *Z. mobilis sacA* encode sucrase; and *E. coli rafD* encodes sucrose hydrolase.

In some cases, the three domains are covalently linked as a single polypeptide, with a total molecular weight of approximately 68,000, and are commonly known as EII. In other cases, domain EIIA, also known as EIII, is a separate polypeptide containing the other two domains (EIIB and -C). These two polypeptides make up an EII-EIII complex. All three domains may exist as three or even four distinct polypeptides (23, 24, 33, 35). The three domains of EII^{Scr} consist of an N-terminal hydrophilic domain, a central hydrophobic transmembrane domain, and a C-terminal hydrophilic domain. These three domains were identified in the predicted amino acid sequence of *S. sobrinus* 6715 EII^{Scr} (Fig. 6).

The N-terminal hydrophilic domain of *S. sobrinus* EII^{Scr} contained a consensus sequence which was identified in the D-glucopyranoside PTS family and centered around Cys-26 (23, 24). This domain is located at the C-terminal end in EII^{Glc} and EII^{Nag} (9, 48). It has been suggested by Lengeler et al. (24) that this region contains the amino acids which interact with the EIII domain and most likely is involved in the phosphorylation of the substrate. It has been demonstrated in EII^{Asg} of *E. coli* that the highly conserved cysteinyl residue is the second phosphorylation site during phosphorylation and translocation of the substrate (29, 30). The central hydrophobic domain contained approximately 350 amino acids and was found to be less homologous to other EIIs. This region probably forms the transmembrane channel (24) and contained a highly conserved consensus sequence centering around a histidine residue (His-316). The GITE motif, the function of which remains unknown, also was observed in this region (amino acids 376 to 379) (24). The C-terminal hydrophilic domain, 170 amino acids in length, corresponded to the well-established $EIII^{Glc}$ domain. During translocation and phosphorylation of PTS substrates, EIIIs recognize HPr and are phosphorylated at a single histidine reside, which is about 80 amino acids away from the C-terminal end. This histidine residue is located within a highly conserved consensus sequence (35). This consensus sequence was found between amino acids 579 to 593 of *S. sobrinus* 6715 EII^{Scr} (Fig. 6) and was identical to that in *S. mutans* GS5 EII^{Scr}. Sequence analysis also indicated that 60% homology existed between *S. sobrinus* 6715 EII^{Scr} and *S. mutans* GS5 EII^{Scr} in this region, whereas much less homology was observed in the rest of the respective predicted EII^{Scr} protein.

A class of interdomain linkers, Q linkers, also was identified in *S. sobrinus* 6715 EII^{Scr}, between the central hydrophobic domain and the C-terminal hydrophilic domain. Q linkers are commonly found in regulatory and sensory transduction proteins in bacteria. They are approximately 20 amino acids in length and are rich in proline, serine, glutamate, arginine, glutamine, and alanine, but do not form a consensus sequence (48, 49). This region has been identified in EII^{Nag} and EII^{Bgl} in which a Pro-Ala-rich sequence was observed (24). It has been postulated that this region may act as a hinge that provides flexibility to the domains (32). A Pro-Ala-rich sequence was observed in the *S. sobrinus* 6715 EII^{Scr} at amino acids 464 to 484, between the central hydrophobic domain and the C-terminal hydrophilic domain. EII^{Scr} from *S. sobrinus* 6715, with a calculated molecular weight of 66,529, was larger than EIII-dependent EII^{Scr}s identified in *E. coli* and *B. subtilis* by approximately 170 amino acids, which is the size of $EIII^{Glc}$ in *Salmonella typhimurium* (28). Homologies between the N-terminal 462 amino acids of *S. sobrinus* EII^{Scr} and *E. coli* or *B. subtilis* EII^{Scr} and between the C-terminal 170 amino acids of *S. sobrinus* EII^{Scr} and *Salmonella typhimurium* $EIII^{Glc}$ also were demonstrated. In addition, the typical C-terminal sequence, a hydrophobic residue followed by two charged residues, that has been identified in EIII-independent EIIs and all EIIIs (35) was observed in *S. sobrinus* 6715 EII^{Scr}. All of these data indicated that EII^{Scr} of *S. sobrinus* is an EIII-independent protein in which the two functional domains have been fused as a single polypeptide.

In a previous study, we identified two *Hin*dIII fragments, 2.7 and 1.1 kb, from *S. sobrinus* 6715 genomic DNA that shared homology with *S. mutans* GS5 *scrA* and *scrB*, respectively, under low-stringency hybridization conditions (5). These two *Hin*dIII fragments were employed as probes in the identification of a 4.2-kb DNA fragment from a λgt10 library of *S. sobrinus* 6715 genomic DNA. The 4.2-kb fragment, when subcloned into an *E. coli-Streptococcus* shuttle vector and transferred to a sucrose-defective derivative mutant of *L. lactis* subsp. *lactis* LM0230, allowed transformants to synthesize EII^{Scr} and sucrose-6-phosphate hydrolase activities and to grow well at the expense of sucrose. However, the results of subsequent sequence analyses of the 4.2-kb DNA fragment indicated that this DNA fragment contained an intact *scrB* and only a partial *scrA*. This truncated N-terminal EII^{Scr} included the N-terminal hydrophilic domain, which contained the highly conserved cysteine residue, and the transmembrane hydrophobic domain, which contained the highly conserved histidine residue, the GITE motif, and the proposed hinge region. It is possible that this truncated EII^{Scr} was able to translocate and phosphorylate the substrate when an additional EIII function was provided by the host (24). The construction of an *L. lactis* subsp. *lactis* LM0230 transformant containing a complete *scrA*, as well as *scrB*, from *S. sobrinus* 6715 is in progress. The availability of such a construct will facilitate the further construction of $scrA^+$ *scrB* and *scrA* $scrB^+$ mutants in *L. lactis* subsp. *lactis* LM0230, which will contribute to our understanding of the functions and regulation of these genes.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant DE08915 to D.J.L. from the National Institute for Dental Research. We thank D. Galli and M. Yeung for helpful discussions.

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